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Short-term Preoperative Dietary Restriction Is Neuroprotective in a Rat Focal Stroke Model

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Abstract

Stroke is a major complication of cardiovascular surgery, resulting in over 100,000 deaths and over a million postoperative encephalopathies annually in the US and Europe. While mitigating damage from stroke after it occurs has proven elusive, opportunities to reduce the incidence and/or severity of stroke prior to surgery in at-risk individuals remain largely unexplored. We tested the potential of short-term preoperative dietary restriction to provide neuroprotection in rat models of focal stroke. Rats were preconditioned with either three days of water-only fasting or six days of a protein free diet prior to induction of transient middle cerebral artery occlusion using two different methods, resulting in either a severe focal stroke to forebrain and midbrain, or a mild focal stroke localized to cortex only. Infarct volume, functional recovery and molecular markers of damage and protection were assessed up to two weeks after reperfusion. Preoperative fasting for 3 days reduced infarct volume after severe focal stroke. Neuroprotection was associated with modulation of innate immunity, including elevation of circulating neutrophil chemoattractant C-X-C motif ligand 1 prior to ischemia and suppression of striatal pro-inflammatory markers including tumor necrosis factor α , its receptor and downstream effector intercellular adhesion molecule-1 after reperfusion. Similarly, preoperative dietary protein restriction for 6 days reduced ischemic injury and improved functional recovery in a milder cortical infarction model. Our results suggest that short-term dietary restriction regimens may provide simple and translatable approaches to reduce perioperative stroke severity in high-risk elective vascular surgery.

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Competing Interests: JRM has consulted with a company that makes medical foods for cancer treatment. This study does not deal with cancer, but stroke, and JRM has no ownership in the aforementioned company (L-Nutra). This does not alter adherence to PLOS ONE policies on sharing data and materials. None of the other authors declares any competing interests.

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Introduction

Perioperative stroke occurring during or soon after surgery is a major cause of morbidity and mortality, with an average incidence of 2–13% in cardiovascular procedures and 0.08–0.7% in non-cardiovascular procedures [1,2]. With 7 million cardiovascular and 21 million non-cardiovascular surgeries performed annually in the US [3] and similar numbers in Europe (extrapolated from data from the Netherlands [4]), annual deaths are calculated to be in excess of 180,000 on these two continents alone. Those left with the debilitating consequences of perioperative stroke/encephalopathy number an order of magnitude higher [5,6]. Treatment of perioperative stroke accounts for a quarter of the resources spent annually for stroke treatment in the USA [5].

Numerous pharmacological compounds have been tested for their ability to provide neuroprotection after stroke, including 5-HT_{1A} agonists, free radical scavengers, immunosuppressants and agents that block excitotoxicity. Despite efficacy in rodent models,

most have failed in clinical trials [7]. While perioperative stroke risk assessment prior to surgery is a common practice, general prophylactic methods are lacking [1,2], underpinning a need for basic research.

Strategies that provide neuroprotection when initiated before the ischemic period are known as preconditioning. Ischemic preconditioning is a phenomenon in which brief periods of ischemia protect against subsequent, longer insults to various organs, including heart [8] and brain [9]. In preclinical models of stroke, ischemic preconditioning prevents subsequent ischemic injury by suppressing the expression of pro-inflammatory cytokines, chemokines, adhesion molecules and transcription factors [10]. Other low-dose stressors such as hypoxia [11], endotoxin [12] or heat shock [13] can also precondition against ischemic injury. However, the clinical application of such methods has remained a matter of debate in large part due to the potential

safety concerns, highlighting a need for safer preoperative prophylactic methods.

Dietary restriction (DR), defined as reduced food intake without malnutrition, extends lifespan and increases resistance to a variety of acute stressors in multiple species, including rodents [14]. Long-term application of DR for 3 months or longer is neuroprotective in rodent models of stroke [15] and excitotoxicity [16–18]. Mechanistically, upregulation of neurotrophic and growth factors, such as brain-derived neurotrophic factor (BDNF) induced by long-term DR [19,20] could be partially responsible for increased protection, as BDNF has been shown to reduce neuronal injury after ischemia [21,22]. Long-term DR also offers benefits against ischemic injury in other organs, such as heart, through a variety of mechanisms including immunosuppression, elevation of reactive oxygen and nitrogen species scavenging mechanisms and upregulation of heat shock protein levels [23,24].

Despite its potential as a safe and effective prophylactic method, the relatively long periods of food restriction employed in preclinical studies (3 months or longer) are not considered feasible in a clinical setting [25]. However, recent data indicate that dietary preconditioning against ischemic injury can be realized in a clinically relevant time frame in rodent models [26]. For example, 2 weeks of 30% reduced daily food intake or 3 days of water-only fasting protect against ischemia reperfusion injury to kidney or liver [27]. Protein restriction in the absence of calorie restriction, or restriction of individual essential amino acids such as tryptophan, can also impart benefits within 6 days [28]. Here, we describe two different pre-operative manipulations – 3 days of water-only fasting and 6 days of protein-free DR – with benefits on focal stroke outcomes in rats.

Methods

Please also see Methods S1 for further details.

Animals

All animal experiments were carried out according to the National Institute of Health (NIH) guidelines for the care and use of laboratory animals and approved by the appropriate local or national ethics board (permit number ESAVI/5459/04.10.03/2011, issued by ELÄINKOELAUTAKUNTA – ELLA Etelä-Suomen aluehallintovirasto, Finland). Adult male Sprague-Dawley (SD) rats weighing 240–300 g were housed under standard conditions with *ad libitum* access to food and water unless indicated otherwise.

Dietary preconditioning regimens

Fasting was performed by removing the complete chow diet (Harlan Teklad Global 2016 Rodent Diet) for 3d while maintaining free access to water at all times. Protein-free dietary restriction was performed by first acclimating all animals to a complete diet made of refined ingredients (Research Diets D12450B) consisting of 18% calories from protein (casein), 72% from carbohydrate (corn starch, maltodextrin, sucrose) and 10% from fat (soybean oil, lard) for 6d. The control group was then maintained on the complete diet, and the protein-free group was given restricted access to an isocaloric diet lacking protein (Research Diets D08043003, consisting of 90% calories from carbohydrate and 10% from fat) at 60% of the average daily intake of the complete diet group for 6d prior to tMCAO and 2d after reperfusion.

Surgical procedures

Two different stroke models were employed. In experiments testing the effects of fasting, a severe focal stroke involving forebrain and midbrain was induced by intraluminal occlusion of the middle cerebral artery (MCA) with a filament for 60 min, followed by reperfusion as described previously [29]. Stroke involving forebrain and midbrain is associated with fever [30]. To determine whether the MCA occlusion surgery produced a lesion, core body temperature in each animal one hour after reperfusion was measured. Experiments evaluating the effect of fasting on severe focal stroke were performed at Charles River Laboratories (CRL), Kuopio, Finland.

In experiments testing the effects of protein-free DR, a mild focal stroke involving cortex only was induced by transient direct occlusion of the right MCA and bilateral CCAs with a 10-0 suture for 60 min followed by reperfusion as described previously [31]. Cortical-only stroke does not result in fever in rats [32] (Figure S1) thus the presence of lesion was verified by behavioral tests up to two weeks post-stroke and/or by TTC staining 48 hours after stroke. Experiments evaluating the effect of protein-free diet on “mild” cortical stroke were performed at the University of Helsinki (UH), Finland.

Behavioral procedures

In the mild suture-induced cortical stroke model, neurological deficits were evaluated using body swing, Bederson's score [33] and cylinder tests [31]; and locomotor activity was measured using an infrared activity monitor (MedAssociates Inc.). All tests were conducted by an investigator blinded to the treatment groups.

Analysis of infarction volume

For determination of infarct volume 7d after severe filament-induced stroke, T2-weighted multi-slice (12–14 continuous slices) MRI images were acquired using a Varian Inova console interfaced to a 4.7T horizontal magnet (MagneX Scientific Ltd, Abington, UK). Lesion quantitation was done by manually delineating total lesion outlines from MRI images based on T2 contrast between lesioned and healthy tissue using MATLAB software by an observer blinded to the treatment groups.

Infarct volume 2d after mild suture-induced cortical stroke was assessed with triphenyltetrazolium chloride (TTC) staining by an observer blinded to the treatment groups as described previously [31].

Blood measurements

Glucose levels were measured from fresh blood with a Glucocard II Super device (Akray Factory Inc., Shiga, Japan). Plasma cytokines were measured on the Rat Demonstration Multi-Spot plate (Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer's instructions. Clinical chemistry analyses from plasma samples were performed with an automatic analyzer (Konelab 30i, Thermo Fisher Scientific, Vantaa, Finland) according to manufacturer's instructions.

Real-time quantitative PCR

Striatum was dissected from snap frozen brains before or 24hr after severe filament-induced stroke for isolation of RNA for cDNA synthesis. Real-time quantitative PCR (qPCR) was performed on a Lightcycler[®]480 real-time PCR system (Roche Diagnostics) using Lightcycler[®]480 SYBR Green I Master complemented with 2.5pmol of primers (Table S1). Reactions were performed in triplicate and analyzed with Lightcycler[®]480 Software. Gene expression was normalized to peptidylprolyl

isomerase A. Similar methods were employed for gene expression analysis from cortex and striatum before or 24hr after mild suture-induced stroke.

Statistics

All values are presented as mean \pm standard error of the mean (SEM). Differences were considered to be statistically significant at the $p < 0.05$ level. Statistical analyses including Student's *t*-test, Mann-Whitney *U*-test and one- or two-way ANOVA or Kruskal-Wallis non-parametric ANOVA followed by appropriate *post hoc* analysis were performed with SPSS 15.0 software.

Results

Neuroprotection against severe focal stroke by preoperative fasting

In hypoxia and global brain ischemia models, short-term water-only fasting is protective [34–38]. However, while global brain hypoperfusion accounts for less than 10% of perioperative strokes in humans [2], the majority (62%) of perioperative strokes are caused by focal ischemic insults that are mechanistically and pathologically different from hypoperfusion. Our first objective was to assess the effect of short-term fasting on severe focal brain ischemia. Rats were subjected to pre-operative water-only fasting for 3d as shown in Fig 1A. As expected, fasted rats exhibited a significant reduction in body weight (Fig 1B). Fasting also significantly reduced blood glucose levels (Fig 1C) and body temperature (Fig 1D) prior to induction of focal stroke by intraluminal occlusion of the middle cerebral artery (MCA) with a filament for 60 min. One hour after reperfusion, body temperature was elevated in both groups, consistent with lesion induction (Fig 1D). Analysis of infarction volumes obtained from T2-MRI images revealed a significant reduction 7d after tMCAO in the fasted group relative to the *ad libitum*-fed group (Fig 1E and F).

Suppression of pro-inflammatory response to severe focal stroke by fasting

Long-term DR is believed to protect against ischemia reperfusion injury at least in part through suppression of inflammatory responses [23]. In the context of neuroprotection, long-term DR is additionally associated with upregulation of neurotrophic factors and growth factors such as BDNF [16,17,19,20], and increased expression of proteins involved in cytoprotection [39]. To gain insight into the molecular mechanisms of protection by fasting against focal stroke, we analyzed striatal gene expression immediately before (baseline) and 24hr after tMCAO using qPCR (Fig 2A). Unlike long-term DR, we found no significant differences in mRNA expression levels of growth factor-related, cytoprotective or pro-inflammatory markers at baseline as a result of 3d of fasting relative to *ad libitum* (AL) fed controls (Fig 2B, Table S1). 24hr after reperfusion, expression of mRNAs encoding for the pro-inflammatory cytokine tumor necrosis factor alpha (TNF α), its receptor TNFRSF1A and its downstream target, the intercellular adhesion molecule 1 (ICAM1) were significantly upregulated in the lesioned hemisphere compared to the control hemisphere in the *ad libitum* group, but not in the fasted group (Fig 2C), suggesting attenuation of the inflammatory response to stroke in fasted rats. Surprisingly, mRNAs encoding for several neurotrophic and growth factors including glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), mesencephalic astrocyte-derived neurotrophic factor (MANF), transforming growth factor beta 1 (TGF β 1), fibroblast growth factor 2 (FGF2) and GDNF family receptor alpha 1 (GFR α 1) were significantly upregulated in the lesioned

striatum after stroke in the *ad libitum* rats but not in the fasted rats (Fig 2C). A similar trend was observed in BDNF mRNA expression, but this effect did not reach statistical significance. Interestingly, no significant differences were observed in the expression of cellular stress response genes (with the exception of HMOX1, Fig 2C) as a function of diet or stroke. The expression of all genes analyzed relative to the reference gene PPIA and normalized to expression in the *ad libitum* group at baseline is provided in Table S1; no significant changes were observed in any of these genes in sham-operated animals 24hr after operation in either dietary group.

Since DR can modulate innate immune activation, we measured the concentration of plasma chemokines/cytokines (CXCL1, IL-1b, IL-4, IL-5, TNF α , IFN γ and IL-13) prior to and 4hrs after tMCAO. Of these, only the pro-inflammatory neutrophil chemoattractant CXCL1 (C-X-C motif ligand 1) was significantly differentially regulated by fasting at baseline, with higher levels in the fasted group than in the *ad libitum* fed group (Fig 2D). Four hours after tMCAO, there was a trend in each of the seven cytokines tested toward being reduced in the pre-fasted group, but none reached statistical significance (Table 1).

Neuroprotection against mild focal cortical stroke by protein-free dietary restriction

Because water-only fasting may in some clinical settings be difficult to tolerate, we next asked whether a milder short-term food restriction regimen could also precondition against stroke. A protein-free DR preconditioning regimen was chosen based on its efficacy in protecting kidney and liver from ischemia reperfusion injury [28].

The intraluminal filament-induced severe stroke model used in the fasting experiments above is one of the most widely used focal stroke models in rats. However, a potential limitation of this method is the severity of the resulting infarction, since stroke lesion of comparable size extending from forebrain to midbrain in humans is most often fatal. We thus continued to probe the potential benefits of protein-free DR using a stroke model that causes a milder lesion restricted to cortex [40], which is a common type of embolic stroke in humans. Our objective was also to assess infarct size at its maximum, i.e. 48 h after stroke and evaluate functional outcome at 2, 7 and 14 days post-stroke.

Rats were acclimated to a complete diet made of refined ingredients for 6d. They were then divided into two groups, balanced for body weight and food intake during the acclimation period (Fig 3A). One group remained on the complete diet for 6d prior to stroke with average *ad libitum* daily food intake of 18.9 g/d. The second group was fed a protein-free diet for 6 d prior to stroke at the reduced amount of 11.4 g/d (~40% calorie restriction). Although the initial aim was to normalize food intake among animals in the short-term protein-free DR group in expectation of food aversion to an incomplete (protein-free) diet, 36% of rats left some protein-free food uneaten, indicative of slightly greater aversion than predicted.

After preconditioning, a mild focal cortical stroke was induced by transient direct occlusion of the right MCA and bilateral CCAs with a suture for 60 min. One hour after reperfusion, body temperatures were not significantly elevated, consistent with milder lesion induction [41] (Fig S1). Two days after stroke induction, animals were sacrificed and infarction volumes were measured by TTC staining of brain sections. Rats in the protein-free DR group showed a 39% reduced infarction volume relative to the *ad libitum* fed group on the complete diet (Fig 3B, C). Similarly, the maximal infarction area was significantly reduced in the protein-free DR group compared to the complete diet group

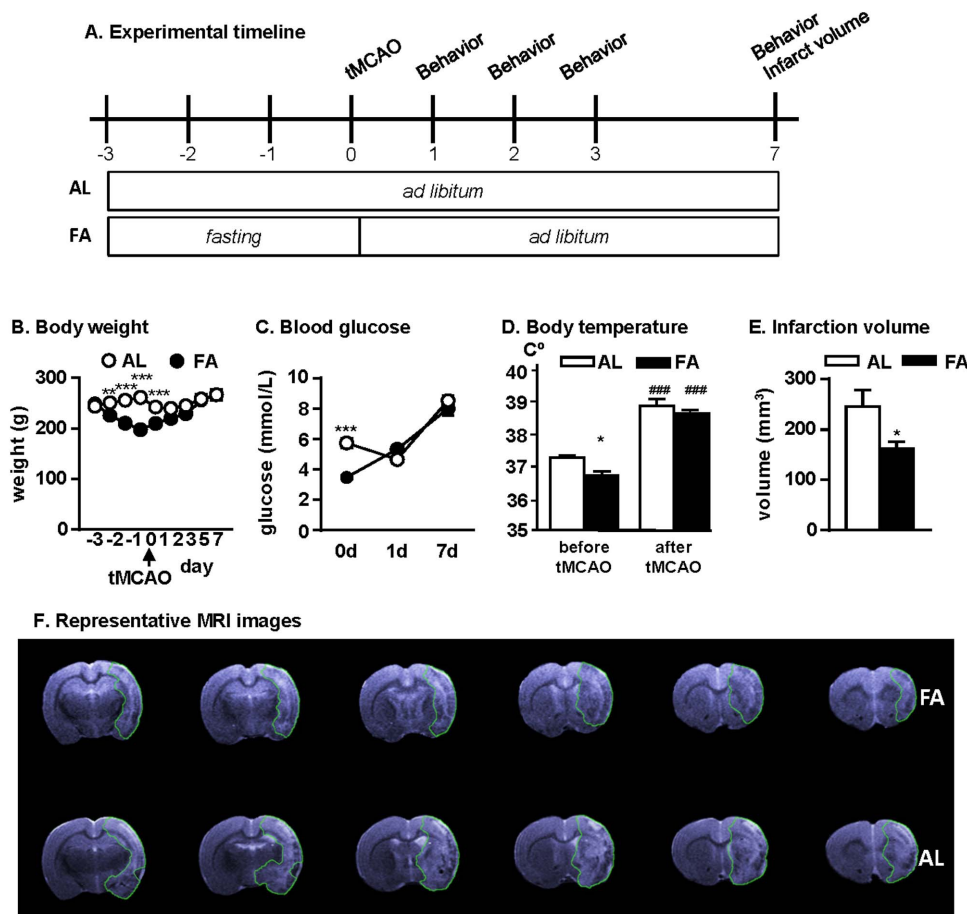


Figure 1. Preoperative 3-day water-only fasting is neuroprotective against stroke. (A) Experimental timeline indicating periods of *ad libitum* feeding and fasting relative to the onset of tMCAO on day 0. AL, *ad libitum* fed ($n = 11$); FA, fasted ($n = 14$). (B) Average body weights prior to and after tMCAO; $F_{8, 23} = 17.69$, $***p < 0.0001$, 2-way ANOVA. (C) Blood glucose levels on the indicated days prior to and after tMCAO; $F_{2, 23} = 14.09$, $***p < 0.0001$, 2-way ANOVA. (D) Body temperature before tMCAO and 1 hour after reperfusion; $***/###p < 0.001$, Student's *t*-test. (E) Infarction volumes at d7 after tMCAO; $*p = 0.0215$, Student's *t*-test. (F) Representative MRI images of the lesioned brain sections with green lines surrounding the lesion.

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(Fig 3D). Prior to sacrifice 2d after stroke induction, rats in the protein-free diet group had smaller behavioral deficits as shown by biased body swing activity (Fig 3E) and Bederson's score (Fig 3F). Although plasma levels of total protein, albumin and urea were reduced in the protein-free DR group at the time of sacrifice consistent with the lack of protein in their diet, a number of other blood parameters including glucose, triglycerides, creatinine and prothrombin were not significantly different between diet groups (Table S2).

Improved functional recovery from mild focal cortical stroke upon short-term protein-free DR

We next assessed whether short-term protein-free DR could promote functional recovery up to 2 weeks after stroke. The experimental diets were applied as described above (Fig 4A). Short-term protein-free DR significantly reduced body weight (Fig 4B). Horizontal locomotor activity on d2 after stroke was significantly improved in the protein-free DR group, indicating faster recovery (Fig 4C). A similar tendency was observed with vertical activity on d2 but did not reach statistical significance (Fig 4D). Body swing activity was significantly reduced in the protein-free DR group on d14 after the stroke compared to the

complete diet group (Fig 4E). Similarly, Bederson's score was significantly reduced on d14 (Fig 4F), with a similar trend on d2 and d7 ($p = 0.0586$ and $p = 0.102$, respectively, Mann-Whitney *U*-test). Rats in the protein-free DR group also showed improved performance in the cylinder test on d14 compared to the complete diet group (Fig 4G).

Finally, in order to shed light on underlying mechanism in comparison to the fasting paradigm, we performed qPCR analysis of gene expression changes in both cortex and striatum 24 hours post-stroke. As expected using this stroke model, gene expression changes in cortex were greater than in striatum. Nonetheless the patterns were similar between these two brain regions, including increased expression of neurotrophic factors BDNF and GDNF, stress response genes HMOX1 and GRP78, and inflammatory markers including ICAM1 and TNFRSF1A (Figure S2). However, with the exception of BDNF in the striatum, changes in expression of these genes in response to stroke were similar between diet groups, suggesting that protection afforded by fasting and protein-free DR could work via different mechanisms or time scale.

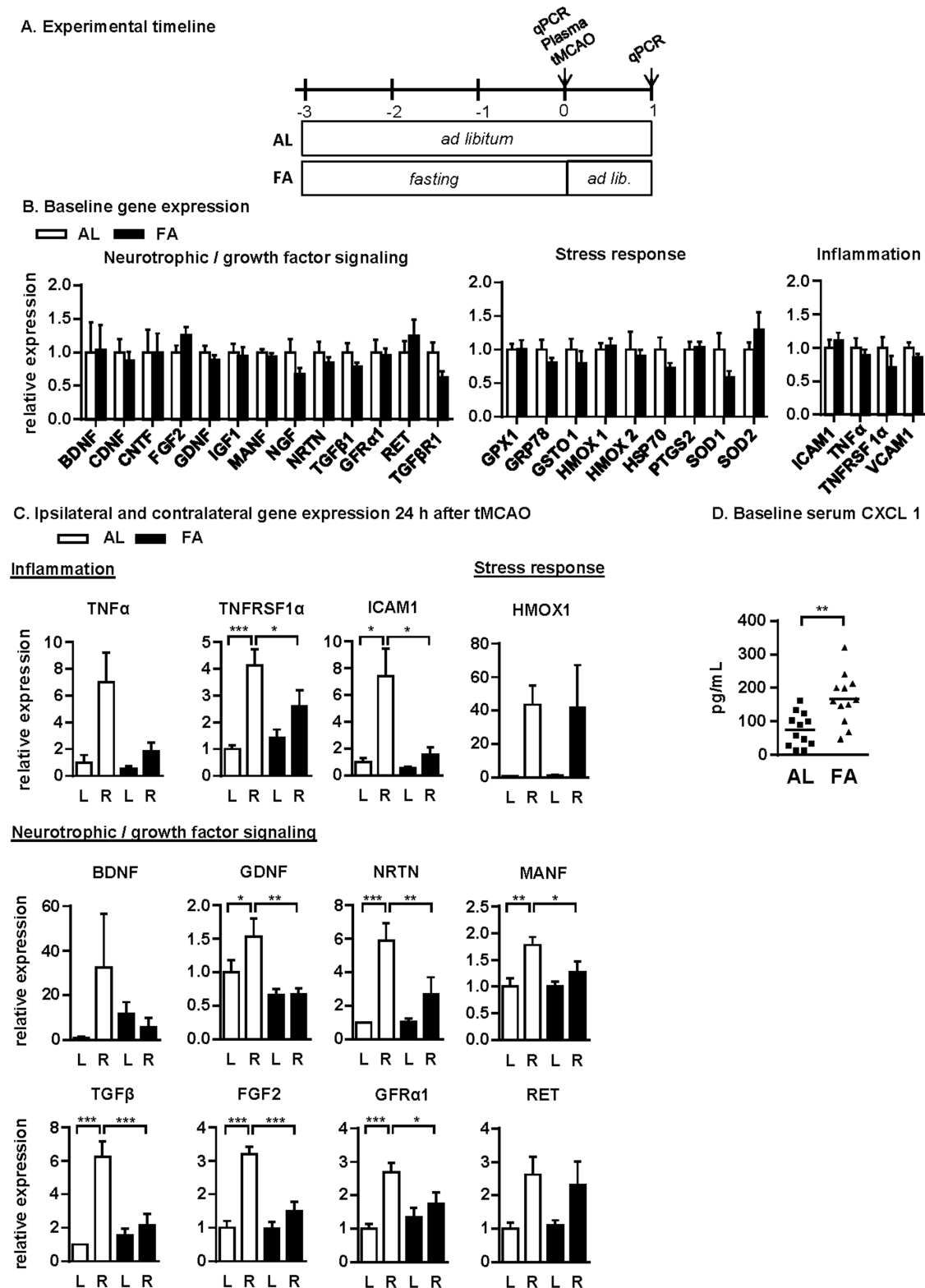


Figure 2. Molecular mechanisms of fasting-induced neuroprotection. (A) Experimental timeline indicating dietary treatments and experimental endpoints relative to tMCAO on day 0. (B) Relative expression of the indicated genes in the striatum of *ad libitum* fed (AL, $n=6$) and fasted (FA, $n=5$) rats at baseline, measured by qPCR and expressed relative to the AL group. (C) Relative expression of selected genes in AL ($n=5$) and FA ($n=6$) rats 24 hours after tMCAO in the unlesioned left (L) and lesioned right (R) striata, measured by qPCR and expressed relative to the unlesioned AL group; $*p<0.05$, $**p<0.01$, $***p<0.001$, 1-way ANOVA. (D) Serum CXCL1 levels in AL ($n=12$) and FA ($n=12$) rats at baseline; $**p=0.0021$, Student's *t*-test.
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Table 1. Plasma cytokine levels (pg/mL) 4 hours after reperfusion in rats fed *ad libitum* (AL) or fasted for 3 days (FA) prior to tMCAO; IFN, interferon; IL, interleukin, CXCL1, C-X-C motif ligand 1; TNF, tumor necrosis factor.

4hr plasma cytokines	IFN α	IL1 β	CXCL1	TNF α	IL4	IL5	IL13
AL (n = 6)	11.7 \pm 3.8	49.3 \pm 9.7	9874 \pm 2069	25.4 \pm 6.3	7.2 \pm 1.7	132.7 \pm 26.1	0.3 \pm 0.3
FA (n = 6)	5.0 \pm 2.1	55.3 \pm 14.3	7205 \pm 2388	12.9 \pm 7.0	5.3 \pm 1.5	87.2 \pm 23.3	0.3 \pm 0.2
p-value	0.16	0.74	0.42	0.22	0.42	0.22	0.94

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Discussion

In hypoxia and global brain ischemia models, short-term water-only fasting is protective [34–38]. However, global brain hypoperfusion accounts for less than 10% of perioperative strokes in humans [2]. The majority (62%) of perioperative strokes are caused by focal ischemic insults that are mechanistically and pathologically different from hypoperfusion. Here we report that three days of preoperative water-only fasting reduced infarct volume compared to *ad libitum* fed rats in a focal stroke model involving forebrain and midbrain. In contrast to long-term DR and ischemic preconditioning, fasting did not increase baseline mRNA expression levels of cellular stress resistance genes such as the molecular chaperones HSP70 and GRP78 [39], or HMOX1 [42]. Nor did it significantly affect the expression of growth and

neurotrophic factors including BDNF and FGF2 [39] or their downstream targets in the brain (Fig 2B, Table S1). Rather, increases in neurotrophic and growth factors and their receptors 24 hours after reperfusion in *ad libitum* fed animals correlated with increased infarct size. This is consistent with the notion that neurotrophic and growth factor upregulation is a relatively late event in ischemic brain damage, occurring downstream of innate immune system activation [43]. Our results are also in line with studies showing that intracranial applications of NTFs are in large part neuroprotective only when injected before the stroke, but are neither neuroprotective nor able to facilitate recovery when applied after the stroke [44]. Taken together, our results suggest that the mechanism of protection by long-term DR and short-term fasting in brain may differ, as has been suggested in other organs including the kidney [27].

In the severe stroke model involving forebrain and midbrain, protection afforded by fasting correlated with an altered inflammatory response. Ischemic injury to thalamic areas causes fever [45], which along with activation of microglia facilitates the expression of pro-inflammatory cytokines such as TNF α [46,47]. Binding of TNF α to its receptor TNFRSF1A on endothelial cells induces ICAM1 expression [48], increasing blood-brain barrier permeability to infiltrating leukocytes and exacerbating tissue damage [49]. In line with the above, we observed a dramatic upregulation of TNF α in the infarcted right hemisphere of *ad libitum* fed animals 24 hours after reperfusion, whereas the expression of TNF α in the lesioned right hemisphere of fasted rats was not significantly affected. TNFRSF1A and ICAM1 expression in the striatum were also both significantly increased after tMCAO in *ad libitum* fed but not fasted rats. Since rats overexpressing TNF α are more susceptible to ischemic injury and mitochondrial dysfunction upon tMCAO [50], while TNF α neutralization is protective [51], our results suggest that suppression of TNF α expression upon focal ischemia reperfusion injury may be an important component of fasting-induced protection from focal stroke.

We also observed a significant increase in the levels of neutrophil chemoattractant CXCL1 in the plasma of rats following a 3d fast. Increased CXCL1 in the blood could reduce local inflammation by reducing the steepness of the chemokine gradient driving neutrophil chemotaxis from the vasculature to the site of brain injury, as well as by affecting adhesion molecule expression on neutrophils themselves [52]. Interestingly, in humans, short-term dietary preconditioning elevates serum levels of IL-8, the human paralogue of CXCL1 [53], warranting further study.

Several physiological parameters can also affect the outcome of experimental stroke. These include body temperature, blood glucose, blood pressure and blood gas levels. Hypothermia has been shown to effectively reduce ischemic injury in experimental models of brain injury [54], and body temperature was slightly but significantly reduced in fasted animals. However, therapeutic

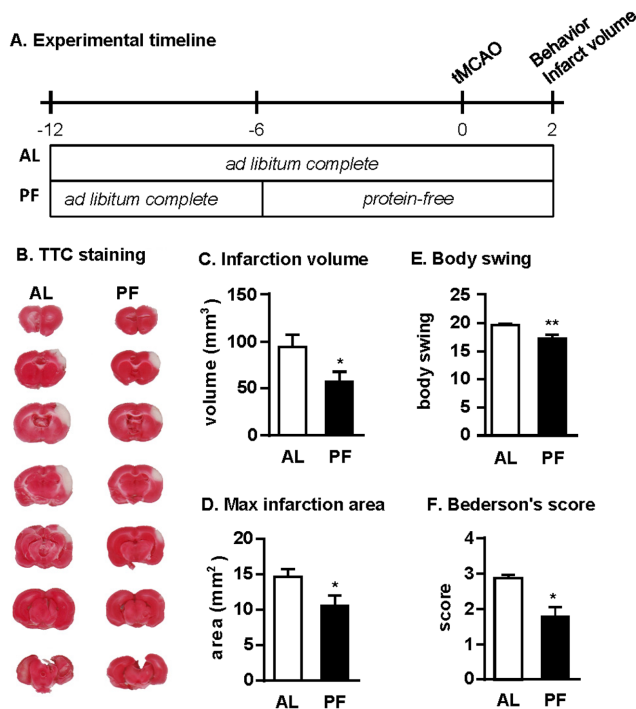


Figure 3. Protein-free DR is neuroprotective against stroke. (A) Experimental timeline indicating periods of *ad libitum* access to a complete diet (AL, n = 15) or restricted access to a protein-free diet (PF, n = 14) relative to the onset of tMCAO on day 0. (B) TTC-stained brain sections showing infarct size (white area). (C) Total infarction volume on d2 after tMCAO; *p = 0.0396, Student's *t*-test. (D) Average maximal infarction area from the slice with the largest infarction area per animal; *p = 0.0320, Student's *t*-test. (E) Biased body swing activity in 20 trials; **p = 0.0016, Mann-Whitney *U*-test. (F) Behavioral performance assessed by modified Bederson's score; *p = 0.0396, Mann-Whitney *U*-test. doi:10.1371/journal.pone.0093911.g003

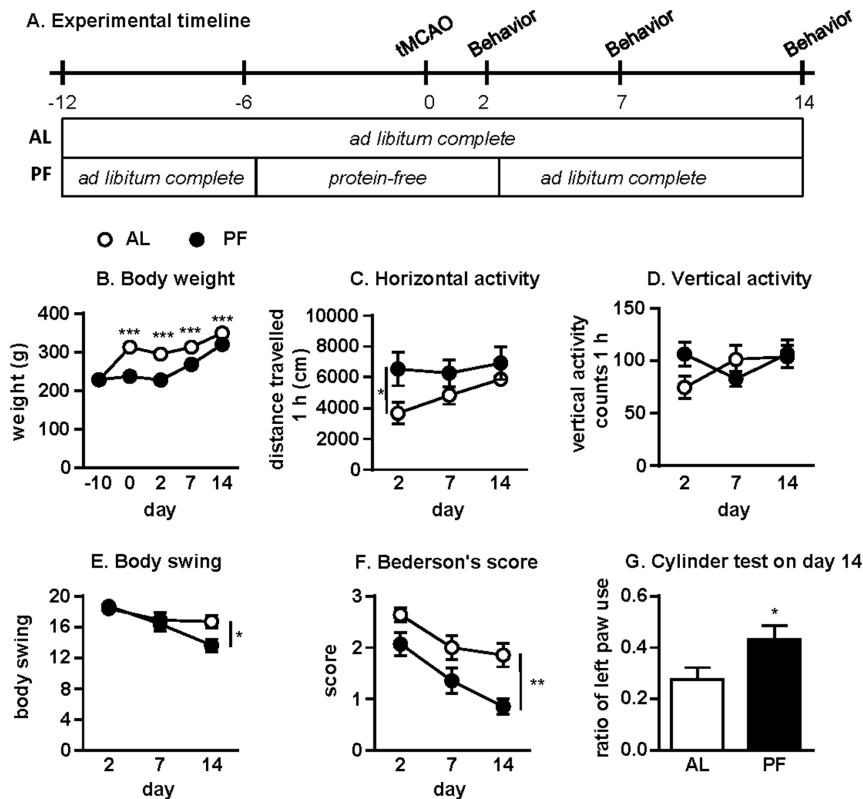


Figure 4. Protein-free DR promotes functional recovery after stroke. (A) Experimental timeline indicating periods of *ad libitum* access to a complete diet (AL, $n=14$) or restricted access to a protein-free diet (PF, $n=14$) relative to the onset of tMCAO on day 0 and subsequent behavioral testing on days 2, 7 and 14. (B) Average body weights on the indicated days relative to tMCAO on day 0; $F_{1,26}=96.20$, $***p<0.0001$, 2-way ANOVA. (C–G) Behavioral tests on the indicated days after tMCAO: (C) horizontal activity; $F_{1,26}=4.994$, $*p=0.034$, 2-way ANOVA; (D) vertical activity; $F_{1,26}=4.150$, $p=0.052$, 2-way ANOVA; (E) biased body swing activity in 20 trials; $*p=0.0211$, Mann-Whitney *U*-test; (F) modified Bederson's score; $**p=0.0018$, Mann-Whitney *U*-test; (G) cylinder test measured on d14 after tMCAO; $*p=0.0492$, Mann-Whitney *U*-test.

hypothermia in rodents and humans is protective only if body temperature is reduced by several degrees [55]. Because fasting reduced body temperature only by a half of a degree, fasting-associated neuroprotection is not likely to be a result of hypothermia. Hyperglycemia and hypoglycemia can both be deleterious after stroke [56,57]. Although baseline blood glucose levels in fasted rats were reduced by 30% relative to the *ad libitum* fed group, this correlated with protection rather than susceptibility to ischemic brain damage. Finally, fasting in rats has been reported not to alter blood pressure or blood gas levels [37], thus making the above parameters unlikely to contribute significantly to protection.

Although water-only fasting is simple and effective in preclinical models of stroke, it may not be well-tolerated in some clinical settings. Thus, the nutritional basis of protection – whether an overall reduction in calories or the removal of specific nutrients – is important not only for understanding the underlying mechanism but also for evaluating the translational potential of the intervention. In fruit flies, the benefits of long-term DR on longevity can be abrogated by the addition of essential amino acids [58]. Long-term protein or individual amino acid restriction can also slow aging in rodents and precondition against acute stressors, including acetaminophen (paracetamol) toxicity [59] and ischemic injury to kidney and liver [28]. In studies of amino acid deficiency-mediated protection against renal and hepatic ischemia reperfusion injury, activation of the GCN2-dependent amino acid starvation response is required for protection in part through

modulation of the systemic inflammatory response to injury and/or by activation of organ-autonomous stress resistance pathways [28]. Taken together, these data suggest broad evolutionary conservation of beneficial adaptive responses to protein/amino acid restriction.

Consistent with these reported benefits of protein/essential amino acid restriction, we found protection with a short-term protein-free DR regimen in a rat model of mild focal stroke, including reduced infarction volume and improved functional recovery. Interestingly, however, preliminary analyses of gene expression changes 24hr after reperfusion to probe candidate mechanisms of protection did not reveal overlap between the protein-free DR and fasting paradigms of neuroprotection. This could be due to different kinetics of gene expression in the severe vs. mild stroke models, or could indicate true differences in underlying mechanisms of protection by fasting and protein-free DR regimens. We focused our gene expression analysis on the 24hr time point in an attempt to uncover primary mechanisms of neuroprotection, as later time points could be confounded by differences in initial lesion size. Nonetheless, inflammatory responses after ischemic brain injury are also time dependent, beginning immediately after injury and lasting for months [60]. Thus, studies using unbiased gene expression analysis at multiple time-points and in different brain areas using gene arrays in combination with physiological, histological and behavioral parameters may help to dissect mechanisms involved in diet-induced neuroprotection and improved recovery after stroke in the

future. How short-term dietary interventions such as fasting or short-term protein-free DR affect ischemic stress resistance in humans is currently unknown. However, there is substantial empirical and observational evidence that medically supervised fasting with periods of 7–21 days is efficacious in the treatment of rheumatic diseases, chronic pain syndromes, hypertension, and metabolic syndrome [61,62]. Because metabolic responses to dietary restriction observed in experimental organisms are shared by humans [63], an expectation that benefits will translate to focal brain ischemia in humans is warranted [26]. The human functional equivalents of the 3d fasting or 6d protein-free DR regimens in rodents tested here are not known, but represent an important next question in translation to the clinic. In conclusion, due to its simplicity, cost-effectiveness and presumed low risk, short-term dietary preconditioning may carry an immediate potential for clinical application in perioperative risk management.

Supporting Information

Figure S1 Rectal body temperature in rat cortical stroke model in *ad libitum* complete (AL, n = 4) and protein free (PF, n = 4) diet groups before and 1 h after reperfusion.

(TIF)

Figure S2 Expression of selected genes 24 hours after tMCAO in the unlesioned left (L) and lesioned right (R) cortices and striata of rats in *ad libitum* (AL, n = 8) and protein-free (PF, n = 7) diet groups, measured by qPCR

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and expressed relative to the unlesioned AL group. Asterisks indicate difference between the lesioned and unlesioned hemispheres in the same diet group. No significant differences were observed between diet groups; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, 1-way ANOVA.

(TIF)

Methods S1 Supplementary Methods.

(DOCX)

Table S1

(PDF)

Table S2 Analysis of the indicated component from blood plasma from rats fed a complete diet *ad libitum* (AL) or a protein free (PF) diet for 6 days prior to and 2 days after induction of cortical stroke.

(DOCX)

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Author Contributions

Conceived and designed the experiments: MA MS JRM JOA. Performed the experiments: KV MA JA SV AP. Analyzed the data: KV MA JA SV JRM JOA. Contributed reagents/materials/analysis tools: MA JRM MS JOA. Wrote the paper: MA JRM JOA.

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